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Characterization of Shiga-like toxin I B subunit purified from overproducing clones of the SLT-I B cistron

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The cistron encoding the B subunit of *Escherichia coli* Shiga-like toxin I (SLT-I) was cloned under control of the *tac* promoter in the expression vector pKK223-3 and the SLT-I B subunit was expressed constitutively in a wild-type background and inducibly in a *lacI*^{q} background. The B subunit was located in the periplasmic space, and less than 10% was found in the culture medium after 24 h incubation. Polymyxin B extracts contained as much as 160 μg of B subunit/ml of culture. B subunit was purified to homogeneity by ion-exchange chromatography followed by chromatofocusing. Cross-linking analysis of purified native B subunit showed that it exists as a pentamer. In gels containing 0.1% SDS the native protein dissociated into monomers. B subunit was found to have the same glycolipid-receptor-specificity as SLT-I holotoxin. Competitive binding studies showed that B subunit and holotoxin had the same affinity for the globotriosylceramide receptor. We conclude that this recombinant plasmid is a convenient source of large amounts of purified SLT-I B subunit, which could be used for biophysical and structural studies or as a natural toxoid.

INTRODUCTION

The Shiga toxin family is a group of closely related subunit toxins produced by *Shigella dysenteriae* serotype 1 and certain strains of *Escherichia coli* [1–3]. SLT-I-producing and SLT-II-producing strains of *E. coli* have been closely associated with haemorrhagic colitis and the haemolytic uraemic syndrome in humans [4,5], and SLT-IIv has been associated with oedema disease of pigs [1,3,6]. In 1977 Konowalchuk, Speirs and co-workers reported that some strains of *E. coli* produced a cytotoxic effect on vero cells in tissue culture. They partially purified the toxins, which were called verotoxins, and showed that several immunologically distinct variants occurred [7]. It has since become apparent that Shiga-like toxins are identical with verotoxins. For instance verotoxin 1 appears to be identical with SLT-I, and verotoxin E to be identical with SLT-IIv [1,3]. Verotoxin 2 produced by the *E. coli* strain E32511 appears to be very similar to but not identical with SLT-II specified by the bacteriophage 933W [8]. Canadian and British workers continue to use the generic term verotoxin whereas Americans use the generic term Shiga-like toxin (SLT). The toxins consist of an A subunit of 32 kDa associated with B subunits of 7.5 kDa. The toxic activity resides in the A subunit and consists of inactivation of protein synthesis due to an *N*-glycosidase activity that removes the adenine base at position 4324 of 28 S rRNA [9]. The B subunit has been shown by cross-linking studies to be present as a pentamer in the native toxin and is responsible for receptor binding [10]. In the case of Shiga toxins SLT-I and SLT-II the receptor has been identified as globotriosylceramide (Gb_3) [11–14]. In the case of SLT-IIv the primary receptor appears to be globotetraosylceramide (Gb_4), even though this toxin also binds Gb_3 [15]. The coding sequences of both subunits are organized as an operon with the A subunit promoter proximal [16]. In the present paper we report the cloning of the coding sequence for the B subunit of SLT-I under the control of the *tac* promoter. The B subunit was purified and found to exist as a pentamer in the native state, and was found to compete effectively with holotoxin for the Gb_3 receptor.

MATERIALS AND METHODS

Bacterial strains, plasmids and media

E. coli TB1 *lac pro rpsL ara thi* ϕ 80d *lacZ* Δ *M15 hsdR* was obtained from Bethesda Research Laboratories (Gaithersburg, MD, U.S.A.). *E. coli* JM101 Δ *lac pro supE thi* (F′ *traD36 lacZ* Δ *M15 pro AB lacI*^{q}) was obtained from Dr. J. D. Friesen (Department of Medical Genetics, University of Toronto, Toronto, Ontario, Canada). Plasmids pTZ18R [17] and pKK223-3 [18] were obtained from Pharmacia (Uppsala, Sweden).

Plasmid pJLB5 consists of a 3.0 kb *Kpn*I fragment of bacteriophage H19B DNA cloned in the *Kpn*I site of pUC18 [19]; see Fig. 1). Fig. 1 summarizes the construction of the overproducing plasmid. To construct plasmid pJLB34, pJLB5 was cut at the *Bgl*II site and digested with nuclease *Bal*31. The ends were filled with Klenow fragment and dNTPs. The fragment remaining after deletion was cleaved with *Eco*RI, and the piece carrying the SLT-I B cistron was purified by agarose-gel electrophoresis. The fragment was recovered from the gel and cloned into pUC18 cut with *Eco*RI and *Hin*cII. The *Eco*RI–*Hin*dIII fragment was cloned in M13mp18 and its nucleotide sequence was determined. The B cistron coding sequence was recovered from pJLB34 as a 1.1 kb *Pst*I fragment and was then cloned in the *Pst*I site of the polylinker of pKK223-3. Clones with the correct orientation of insertion relative to the *tac* promoter were identified by restriction-endonuclease analysis. One plasmid with the correct orientation was selected and designated pJLB120. pJLB120 was transformed into *E. coli* TB1 for constitutive expression and into

Abbreviations used: SLT-I, *Escherichia coli* Shiga-like toxin I; SLT-II, *Escherichia coli* Shiga-like toxin II; SLT-I B, Shiga-like toxin I binding subunit; Gb_3, globotriosylceramide (Galα1–4Galβ1–4Glc-ceramide); Gb_4, globotetrosylceramide (GalNAcβ1–3Galα1–4Galβ1–4Glc-ceramide; GM_1, Galβ1–3GalNAcβ1–4(NeuAcα2–3)Galβ1–4Glc-ceramide; GM_2, GalNAcβ1–4(NeuAcα2–3)Galβ1–4Glc-ceramide; MAb, monoclonal antibody.

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E. coli JM101 for inducible expression. Bacteria were grown in L-broth [20] or brain heart infusion broth (Difco Laboratories, Detroit, MI, U.S.A.) supplemented as necessary with carbenicillin at 50 μg/ml and isopropyl β-D-thiogalactoside (Bethesda Research Laboratories) at 1 mM.

DNA manipulations

Restriction endonucleases and T_4 DNA ligase were purchased from Boehringer Mannheim Biochemicals (Montreal, Quebec, Canada). Plasmid DNA was prepared by the method of Birnboim & Doly [21]. DNA fragments were purified from low-melt-agarose-gel electrophoresis or polyacrylamide-gel electrophoresis and were recovered with the Geneclean Tm kit (Biocan Scientific, Mississauga, Ontario, Canada) or by electroelution [22] respectively.

Ligations were carried out overnight at 14 °C or at room temperature for 4–6 h, with the use of 2 units of T_4 DNA ligase per 20 μl reaction mixture. Nucleotide sequencing was performed by the Sanger dideoxy chain-termination method [23] with the Sequenase Tm version 2.0 kit from U.S. Biochemical Corp. (Cleveland, OH, U.S.A.).

Expression of SLT-I B subunit

For *E. coli* JM101 (pJLB120), an overnight culture was used to inoculate fresh L-broth supplemented with carbenicillin (50 μg/ml) and was grown to mid-exponential phase (A_{600} = 0.3–0.6) at 37 °C, with shaking at 300 rev./min. Isopropyl β-D-thiogalactoside was added to a final concentration of 1 mM, and incubation was continued with aeration. For *E. coli* TB1 (pJLB120), an overnight culture was used to inoculate fresh L-broth supplemented with carbenicillin (50 μg/ml), and this was grown for 12–18 h at 37 °C, with shaking at 300 rev./min. In both cases the culture was harvested and the pellet was washed once with phosphate-buffered saline (0.15 M-NaCl/10 mM-sodium phosphate buffer, pH 7.4) before extraction.

Polymyxin B extraction

The washed pellet was resuspended in phosphate-buffered saline containing 0.1 mg of polymyxin B/ml in one-quarter of the original culture volume and extracted as previously described [24]. For the purpose of purification, 18 h cultures of *E. coli* TB1 (pJLB120) were extracted with polymyxin B and the extracts were concentrated 10-fold by using a stirred-cell Amicon concentrator with a Ym-5 membrane (Amicon Corp., Danvers, MA, U.S.A.).

Membrane extracts

E. coli TB1 (pJLB120) was grown to mid-exponential phase (A_{600} = 0.4). After the cells had been harvested and the pellet washed with 10 mM-Tris/HCl buffer, pH 8.0, they were resuspended in the same buffer and broken by sonication for 90 s in an MSE Ultrasonic Disintegrator (Johns Scientific, Toronto, Ontario, Canada). Unbroken cells were removed by centrifugation at 7700 *g* for 10 min and the supernatant was centrifuged at 43500 *g* (Sorvall SS34 rotor) for 120 min at 4 °C. The pellet was resuspended in distilled water [25].

Protein determination

Protein determinations were performed with the BCA protein assay reagent (Pierce Chemical Co., Rockford, IL, U.S.A.) according to the method of Smith *et al.* [26], with BSA as standard.

PAGE

SDS/PAGE was performed by the method of Laemmli [27] and Tricine/PAGE according to the method of Schägger & von Jagow [28]. Samples were loaded either in SDS loading buffer [4% (w/v) SDS in 50 mM-Tris/HCl buffer, pH 6.8, containing 2% (v/v) 2-mercaptoethanol and 10% (v/v) glycerol] or in a modified buffer containing no 2-mercaptoethanol. Molecular-mass standards were obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.) or Sigma Chemical Co. (St. Louis, MO, U.S.A.). Gels were stained with Coomassie Blue R-250 (Bio-Rad Laboratories) or by the silver stain method of Merril *et al.* [29].

Purification of MAb 13C4

The hybridoma line 13C4 was obtained from the American Type Culture Collection (A.T.C.C. no. CRL 1794) and produces an antibody directed against the B subunit of SLT-I as previously described [30]. Cells were grown in RPMI 1640 medium (Gibco, Burlington, Ontario, Canada) supplemented with 10% (v/v) fetal-calf serum, 10 mM-Hepes and 1 mM-sodium pyruvate in 1-litre spinner flasks (Bellco), and MAb 13C4 was purified from culture supernatants with an Affi-Gel Protein A MAPS II kit (Bio-Rad Laboratories) as recommended by the manufacturer.

Western-blot analysis

Western blots were performed as described by Towbin *et al.* [31]. Protein that had been transferred to nitrocellulose filters (Schleicher and Schuell, Keene, NH, U.S.A.) was allowed to react with MAb 13C4. The washed filters were then incubated with peroxidase-conjugated anti-(mouse IgG) antibody (Bio-Rad Laboratories) and developed with chloro-1-naphthol substrate.

Quantification of SLT-I binding subunits

This was performed by a modification of the method of Hawkes *et al.* [32]. Periplasmic extracts of SLT-I B-producing clones, prepared by polymyxin B extraction, were diluted as required and were filtered on to nitrocellulose paper in a slot-blot apparatus (Bio-Rad Laboratories). SLT-I B was detected by using MAb 13C4 according to the Western-blot procedure described above. Blots were scanned with a Molecular Dynamics model 300A computing densitometer. SLT-I B was quantified by comparison with a standard curve generated with purified B subunit protein.

Periplasmic extracts of *E. coli* TB1 containing the vector pKK223-3 with no insert gave a completely negative result in the slot-blot assay. The results of assays of purified B subunit diluted in periplasmic extracts of *E. coli* TB1 (pKK223-3) did not differ significantly from those of the same amount of B subunit diluted in phosphate-buffered saline. There was a significant variation in the assay, which contributed significantly to the large standard deviations in the amount of B subunit detected. Thus when a single sample was assayed ten times the standard deviation was 20% of the mean. Nevertheless when the same sample was assayed on separate days there was good agreement in the results.

Purification of SLT-I B

The concentrated polymyxin B extract was dialysed overnight against 50 mM-Tris/HCl buffer, pH 7.4, and then applied to a DEAE-Sephacel column (1 cm × 20 cm) equilibrated with 50 mM-Tris/HCl buffer, pH 7.4. Bound material was eluted by using a linear gradient of 0–1 M-NaCl in 50 mM-Tris/HCl buffer, pH 7.4, and 5 ml fractions were collected. Fractions containing SLT-I B were identified, pooled and concentrated with Centriprep-3 concentrators (Amicon Corp.). This pool was dialysed overnight against 25 mM-imidazole/HCl buffer, pH 7.4, and was applied to a column (1.5 cm × 20 cm) of Polybuffer exchanger 94 (Pharmacia) equilibrated with the same buffer. Elution was

carried out with a degassed solution of Polybuffer 74 (Pharmacia) diluted 1:8 with distilled water and adjusted to pH 4.0 with HCl (11 column volumes). Fractions (5 ml) were collected, and the B subunit-positive fractions were pooled and concentrated with Centriprep-3 (Amicon), and ampholytes were removed by means of a Sephadex G-50 gel-filtration column.

Radiolabelling of SLT-I

SLT-I was purified as previously described [24] and iodinated by using Iodobeads (Pierce Chemical Co.) as described by the manufacturer. Two Iodobeads were added to 1 mCi of $Na^{125}I$ in 180 μl of 0.1 M-sodium phosphate buffer, pH 7.2, and were incubated for 5 min. The reaction was initiated by adding 20 μl of SLT-I (1 mg/ml) and was allowed to proceed for 10 min. The labelled protein was separated from unbound I_2 by gel filtration on a Sephadex G-25 column.

Preparation of Gb_3-coated plates

Purified Gb_3, cholesterol and phosphatidylcholine were mixed together in chloroform/methanol (2:1, v/v) in the proportions 2:10:5 (by wt.) and dried down under a stream of N_2. The residue was resuspended in methanol to give a Gb_3 concentration of 1 μg/ml. Portions (100 μl) were dispensed into wells of flexible micro-titre plates (Falcon). The methanol was allowed to evaporate overnight, and the surface of the coated wells was blocked with 2% (w/v) BSA in phosphate-buffered saline for 1 h. The wells were finally washed four times with phosphate-buffered saline containing 0.2% BSA before use.

Competitive binding assay

Serial dilutions of SLT-I (1 mg/ml) or purified B subunit (1 mg/ml) were made in phosphate-buffered saline containing 0.2% BSA and 0.04% NaN_3, and ^{125}I-SLT-I was added to each dilution to give a final value of 10^5 c.p.m./well. After mixing, 100 μl of each dilution was pipetted in triplicate into Gb_3-coated wells and was incubated for 2 h at room temperature. Wells were washed five times with the above buffer, then clipped from the plate and the radioactivities counted on an LKB 1282 γ-radiation counter. The ability of each SLT-I or SLT-I B dilution to inhibit ^{125}I-SLT-I binding was expressed as a percentage of radioactivity bound when no competitor was present (percentage of maximum binding).

T.l.c. binding assays were performed as previously described by Lingwood *et al.* [13].

Gel-filtration (h.p.l.c.) analysis of SLT-I B subunit

A 16 μg portion of purified SLT-I B was injected on to two Waters Protein-Pak 125 h.p.l.c. columns (each 30 cm × 4.6 mm internal diam.) connected in series and eluted at 0.5 ml/min with phosphate-buffered saline. Protein elution was monitored by A_{280} readings. Before the injection of SLT-I B, the columns were calibrated with Blue Dextran (void volume, V_0), BSA, ovalbumin, carbonic anhydrase, cytochrome *c* and aprotinin, all from Sigma Chemical Co. The elution volume (V_e) of each standard was determined and log (molecular mass) plotted against V_e/V_0 to yield a standard curve, from which the apparent molecular mass of SLT-I B could be calculated.

Cross-linking of SLT-I B subunit

B subunit was cross-linked with dimethyl suberimidate as described by Davies & Stark [33]. Cross-linked material was analysed by Tricine/PAGE.

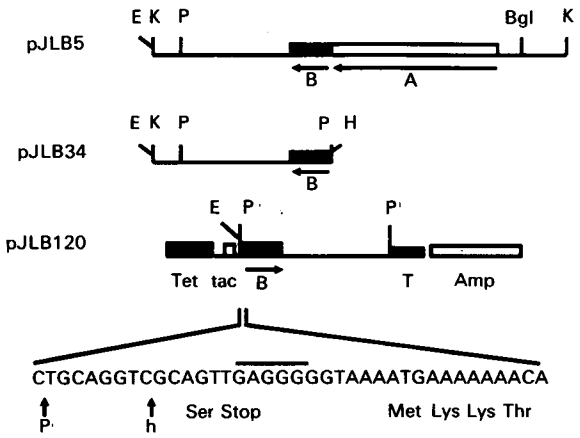

Fig. 1. Construction of the SLT-I B clone pJLB120

Insertion of the B subunit coding sequence into the *Pst*I site of pKK223-3 adjacent to the *tac* promoter is shown. Also indicated are the Shine–Dalgarno sequence (bar above the sequence), the 3′-terminal codon of the A subunit, and the first four codons of the B subunit signal sequence. Horizontal arrows show direction of transcription. Abbreviations: E, *Eco*RI; K, *Kpn*I; P, *Pst*I; Bgl, *Bgl*II; H, *Hin*dIII, h, *Hin*cII; T, rrnb terminator; Amp, β-lactamase gene; Tet, tetracycline-resistance sequence; tac, *tac* promoter.

RESULTS

Construction of the B-subunit-overproducing plasmid pJLB120

The construction is shown in Fig. 1 and is described in the Materials and methods section. *Bal*31 nuclease deletion, fortuitously, gave rise to a clone pJLB34. Sequencing of this clone showed that the deletion ended in the 3′-end of the A subunit cistron. The B subunit coding sequence could be cleaved from pJLB34 as a *Pst*I fragment and was inserted into the *Pst*I site of the expression vector pKK223-3, which contains the *tac* promoter upstream of a polylinker [18]. The sequence of the 5′-end of the *Pst*I fragment was determined in pTZ18R and is shown in Fig. 1. It can be seen that one codon of the 3′-terminus of the A cistron preceded the 12 bp intercistronic gap and that the Shine–Dalgarno sequence and start codon of the B cistron are retained.

Expression of SLT-I B subunit by *E. coli* TB1 and JM101

B subunit was expressed in both strains JM101 (pJLB120) and TB1 (pJLB120), and periplasmic extracts were prepared by using polymixin B. These extracts were concentrated with Centricon-3 ultrafiltration units (Amicon Corp.), and samples were subjected to SDS/15%-PAGE. Fig. 2 shows that both clones expressed the SLT-I B subunit gene, as evidenced by protein bands corresponding to that of purified SLT-I B subunit. In the case of *E. coli* TB1 (pJLB120) the extracts are from cultures grown for 18 h and represent the equivalent of 72 μl of cell culture (lane B). The same quantity of *E. coli* JM101 (pJLB120) extract was loaded in lane C; however, this culture was grown for only 3 h after induction. Confirmation of SLT-I B production was provided by Western-blot analysis of the identical SDS/PAGE gel with the use of MAb 13C4 (lanes F and G respectively).

Efficiency of extraction and localization

The efficiency with which SLT-I B could be extracted by polymyxin B from cells was assessed by means of the slot-blot assay. An overnight culture of *E. coli* TB1 (pJLB120) was harvested, and the pellet was initially extracted with polymyxin B for 1 h, followed by re-extraction for 30 min. After the second extraction the pellet was washed for 30 min with phosphate-

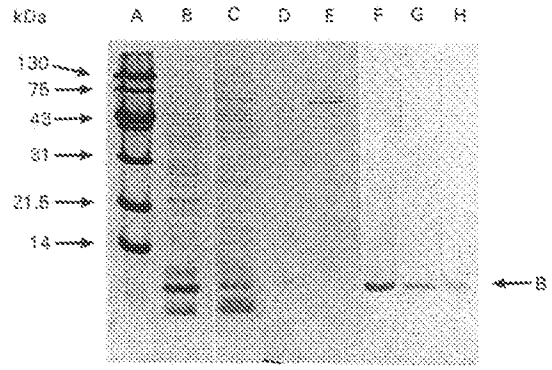

Fig. 2. SDS/PAGE (lanes A–E) and Western-blot analysis (lanes F–H) of periplasmic extracts of *E. coli* TB1 (pJLB120) and *E. coli* JM101 (pJLB120)

Lanes A–E of the gel were stained with 0.1% Coomassie Blue. Lanes F–H of an identical gel were transferred to nitrocellulose paper and allowed to react with MAb 13C4. Lane A, molecular-mass markers; lane B, *E. coli* TB1 (pJLB120); lane c, *E. coli* JM101 (pJLB120); lane D, purified B subunit; lane E, *E. coli* TB1 (pKK223-3), vector control; lane F, *E. coli* TB1 (pJLB120); lane G, *E. coli* JM101 (pJLB120); lane H, purified B subunit. The molecular-mass standards were phosphorylase *b* (130 kDa), BSA (75 kDa), ovalbumin (43 kDa), carbonic anhydrase (31 kDa), soya-bean trypsin inhibitor (21.5 kDa) and lysozyme (14 kDa).

buffered saline at 37 °C, and the wash was collected. The supernatant of the harvested culture was also assayed to determine whether SLT-I B was being excreted into the culture medium.

In five separate experiments, the first two polymyxin B extracts yielded SLT-I B at quantities of 80 ± 58 and 48 ± 25 μg/ml of the original culture respectively. More significantly, however, the phosphate-buffered saline wash of the pellet contained residual SLT-I B at 39 ± 32 μg/ml of culture. The mean total yield from polymyxin B extracts and pellet wash was 160 ± 79 μg/ml of culture. The culture .supernatant, when assayed, contained 8 μg/ml of culture, indicating that less than 10% of SLT-I B was being secreted into the culture medium. Since the pellet after extraction still contained relatively high residual quantities of SLT-I B, membrane preparations were examined to determine whether the protein was being trapped in the membrane. The yield from membrane analysis was 0.31 μg/ml of culture.

Production of SLT-I B subunit during the growth cycle

Production of the B subunit by *E. coli* JM101 (pJLB120) and *E. coli* TB1 (pJLB120) was assessed during their growth cycle. At specified intervals portions of the cultures were harvested and the pellets subjected to a single extraction with polymyxin B (0.1 mg/ml). The extracts were then analysed by the slot-blot assay. Fig. 3 shows the quantities of B subunit produced over time for the two strains. The expression of B subunit by *E. coli* TB1 (pJLB120) increased during the first 6 h of growth, corresponding to exponential and early stationary phases. In the case of *E. coli* JM101 (pJLB120), negligible quantities of SLT-I B were produced before induction, after which expression of the protein increased throughout the exponential and early stationary phases. Comparison of the two strains showed that *E. coli* JM101 (pJLB120) expressed greater quantities of SLT-I B than did *E. coli* (pJLB120). For example, after 6 h B subunit in the former was at a level of 100 μg/ml compared with 63 μg/ml in the latter. In addition, both strains demonstrated a decrease in the concentration of B subunit after 24 h. This decrease was sharper in *E. coli* JM101 (pJLB120) than in *E. coli* TB1 (pJLB120).

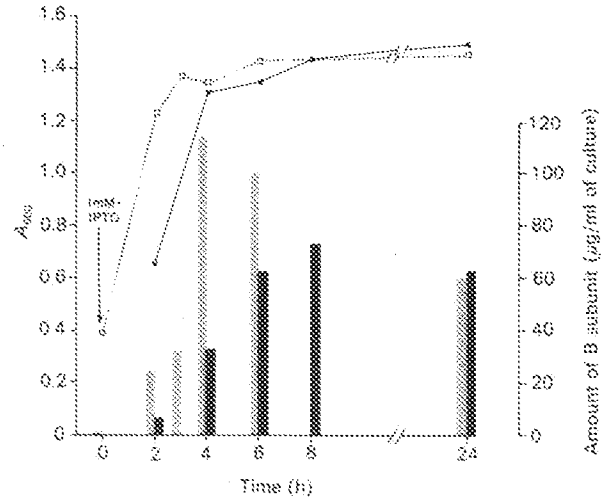

Fig. 3. Quantification of SLT-I B subunit produced in periplasmic extracts of *E. coli* TB1 (pJLB120) and *E. coli* JM101 (pJLB120) during their growth cycle

The amount of B subunit is expressed as μg of B subunit/ml of culture and is represented by stippled bars [▨; *E. coli* JM101 (pJLB120)] and black bars [■, *E. coli* TB1 (pJLB120)]. The A_{600} of the cultures is represented by the continuous line [●—●; *E. coli* TB1 (pJLB120)] and the broken line [□···□; *E. coli* JM101 (pJLB120)]. The arrow indicates the time at which isopropyl β-D-thiogalactoside (IPTG) was added to the *E. coli* JM101 (pJLB120) culture.

Purification

DEAE–Sephacel chromatography of concentrated periplasmic extract yielded a single protein peak containing the B subunit eluted at 200 mM-NaCl (results not shown). Application of the pooled B-subunit-containing fractions to a chromatofocusing column and elution with a pH gradient of 7.4–4.0 yielded a peak that was eluted at pH 5.9 and that contained the B subunit (Fig. 4). Gel filtration of native B subunit showed a single molecular-mass species. Amino acid analysis results (Table 1) showed a single protein species with a composition almost identical with that predicted by the nucleotide sequence.

Purified B subunit appeared to be homogeneous when assessed by silver-stained SDS/PAGE gels (results not shown). When the gel was overloaded and stained with Coomassie Blue it became apparent that most of the material migrated at 7 kDa, while a faint band corresponding to a 15 kDa species was also seen (Fig. 5). The band at 15 kDa was eliminated when the sample was denatured but not reduced. It was suspected that the 15 kDa material represented a dimeric species of B subunit. This was confirmed by Western blotting, which showed that the 15 kDa species reacted with a B-subunit-specific monoclonal antibody PH-1 (Fig. 5).

The molecular mass of native purified B subunit was assessed by h.p.l.c. gel filtration. A calibration curve was generated by plotting log(molecular mass) against V_e/V_0 for the standards BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), cytochrome *c* (12 kDa) and aprotinin (6.5 kDa). The B subunit was eluted as a single peak at a position corresponding to a molecular mass of 26 kDa, suggesting that it was a multimer. Cross-linking analysis suggested that native B subunit exists as a pentamer (Fig. 6). SDS/PAGE of native B subunit showed that it co-migrated with reduced boiled B subunit, suggesting that the pentamer dissociated into monomers in the presence of 0.1% SDS alone (results not shown).

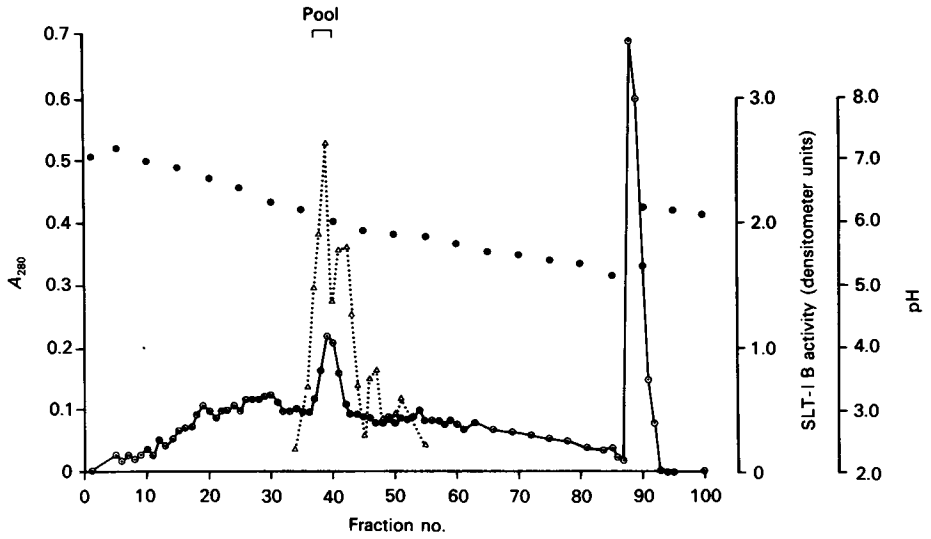

Fig. 4. Chromatofocusing of partially purified SLT-I B

Fractions from DEAE-Sephacel salt elution containing the B subunit were dialysed against equilibration buffer and chromatofocused as described in the Materials and methods section. Fractions (5 ml) were collected, and A_{280} (⊙—⊙), SLT-I B activity (by slot-blot; △···△) and pH (● ●) were determined.

Table 1. Amino acid composition of purified SLT-I B subunit

Amino acid analysis was performed after 24 h hydrolysis of SLT-I B. Abbreviation: N.D., not determined.

Amino acid	Composition (mol of residue/mol)	
	Expected	Observed
Asx	10	10.2
Glx	5	5.8
Ser	3	2.5
Gly	6	6.2
His	1	1.0
Arg	2	2.1
Thr	10	9.4
Ala	2	2.4
Pro	1	1.2
Tyr	2	2.0
Val	6	5.8
Met	1	1.0
Cys	2	N.D.
Ile	3	2.5
Leu	5	5.1
Phe	4	4.0
Lys	5	4.8

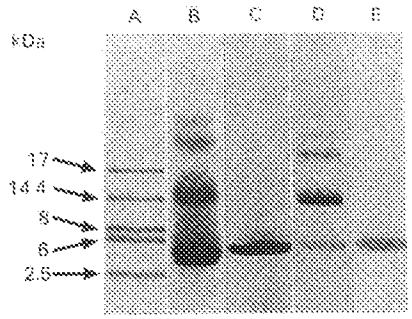

Fig. 5. SDS/Tricine/PAGE (lanes A–C) and Western-blot analysis (lanes D and E) of purified SLT-I B subunit

Samples were dissolved in SDS sample buffer containing 2-mercaptoethanol (lanes A, B and D) or no 2-mercaptoethanol (lanes C and E). Lanes A–C were stained with Coomassie Blue. Lanes D and E were transferred to nitrocellulose paper and allowed to react with the anti-(SLT-I B) monoclonal antibody PH-1 (supplied by Dr. C. Lingwood). The molecular-mass markers (lane A) were myoglobin polypeptide backbone (17 kDa), myoglobin fragments I+II (14.4 kDa), myoglobin fragment I (8 kDa), myoglobin fragment II (6 kDa) and myoglobin fragment III (2.5 kDa).

Glycolipid binding

In the t.l.c. binding assay, purified SLT-I B had the same binding specificity as SLT-I holotoxin, that is it bound to Gb_3 but not to lactosylceramide, digalactosyldiacylglycerol or Gb_4 (results not shown). In addition, no binding was detected to GM_1, GM_2, monogalactosyldiacylglycerol, galactosylceramide and Forssman antigen (results not shown). To determine whether purified B subunit had the same affinity for Gb_3 as had SLT-1 holotoxin, we used a competitive radiobinding assay. Purified B subunit competed in a concentration-dependent manner with an affinity that was not statistically different from that of SLT-I, as shown by the coincident competition curves in Fig. 7.

DISCUSSION

The B subunit of SLT-I has an amino acid sequence identical with that of Shiga toxin [16]. Both have been shown to bind to the glycolipid Gb_3 [11–13], and all the evidence suggests that this is the physiological receptor that mediates the fluid response in rabbit small-intestinal loops [34] and the cytotoxic response in HeLa cells [35] and Daudi lymphoma cells [36]. Furthermore B-subunit-specific monoclonal antibodies have been shown to have neutralizing activity [30]. A synthetic peptide composed of residues of the B subunit of SLT-I/Shiga toxin was shown to induce a neutralizing antibody response in rabbits [37]. Although several investigators have succeeded in producing pure B subunit by dissociating purified holotoxin [10,38,39], the recombinant plasmid described in the present paper provides a convenient source of large amounts. Furthermore the strain is not a

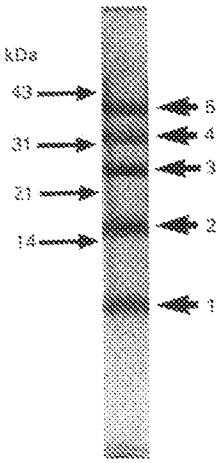

Fig. 6. Cross-linking analysis of SLT-I B subunit

Purified B subunit cross-linked with dimethyl suberimidate was electrophoresed in a 16.5% polyacrylamide gel with Tricine buffer. Arrow 1 indicates the migration of the monomeric species, and arrows 2, 3, 4 and 5 indicate dimers, trimers, tetramers and pentamers respectively. Positions of molecular-mass markers are indicated on the left.

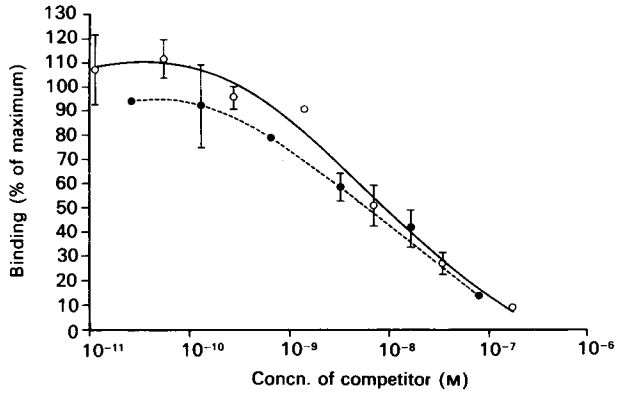

Fig. 7. Competition binding of SLT-I and SLT-I B subunit to Gb_3

Binding of labelled SLT-I in the presence of unlabelled inhibitor is expressed as the percentage of total binding in the absence of inhibitor and is plotted against inhibitor concentration. The curves show inhibition by unlabelled holotoxin (●---●) and by purified B subunit (O—O).

biohazard. B subunit can, then, easily be purified from periplasmic extracts, either as described here, or by the single step P1 glycoprotein affinity chromatography method recently described by Donohue-Rolfe *et al.* [40].

Our results demonstrate that the B subunit is predominantly localized to the periplasmic space, since 10 times more was found in periplasmic extracts than in the culture supernatant. There was a surprisingly large standard deviation when five separate cultures were each subjected to two rounds of polymyxin B extraction followed by one wash in phosphate-buffered saline. Clearly part of the variation was due to the relatively large standard deviation of 15–20% that was found for the slot-blot assay itself. However, it was also clear that the amount of B subunit obtained at each extraction varied considerably. Thus in some cases almost all the B subunit was obtained with the first polymyxin B extraction whereas in other cases the same amount was obtained at each of the two polymyxin B extractions and the phosphate-buffered saline wash. We therefore consider that further efforts to refine and standardize the extraction technique will be important to obtain uniformly high yields. It is also possible that there was significant variation in the production of B subunit in different cultures, but we have not studied this in detail. The results show that induction of expression in the *lacI*^{q} host JM101 with isopropyl β-D-thiogalactoside results in the production of about 50% more B subunit than is produced constitutively in the TB1 host. Since isopropyl β-D-thiogalactoside and a special induction protocol were unnecessary, and the yield was still high, we found it more convenient and less expensive to use the constitutively overproducing strain TB1 (pJLB120). We have consistently been able to obtain 100–120 mg of purified B subunit from 8-litre cultures (J. Gariepy & B. Boyd, unpublished work).

The molecular mass of 26 kDa obtained by gel filtration is clearly lower than expected for a pentamer. Additional biophysical analysis is required to investigate this discrepancy. The cross-linking data show that the SLT-I B subunit exists as a pentamer in the native state in the absence of the A subunit. Our results differ from those reported by Donohue-Rolfe *et al.* [39], who found that B subunit produced by denaturation of holotoxin appeared to exist as a monomer on the basis of cross-linking studies. We believe that their results may have been due to failure to renature the purified B subunit completely. The existence of SLT-I B subunit as a pentamer is similar to the B subunit of cholera toxin [41]. However, native *E. coli* heat-labile enterotoxin and cholera toxin B subunit pentamers are stable in SDS/PAGE [41], whereas even non-reduced native SLT-I B subunit migrated as a monomer in the presence of SDS in our studies. We wondered whether association with the A subunit would modulate the binding specificity or affinity of the B subunit pentamer. Our results clearly show that the specificity for the Gb_3 receptor is retained by the B subunit. Similarly, the competition studies show that the affinity for Gb_3 is virtually identical. This suggests that purified B subunit could be used as a convenient non-toxic marker to examine the routing of toxin in animal models and as a reagent to identify toxin-binding sites in tissue sections *in vitro*. It will also be interesting to see whether purified B subunit can be used as a natural toxoid for immunization. Migasena *et al.* have demonstrated that the B subunit of cholera toxin in combination with killed whole vibrios, when given orally, was immunogenic [42]. It is conceivable that such immunization could be used to prevent haemolytic uraemic syndrome, especially in Argentina, where the incidence appears to be very high [43].

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